PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A1

(11) International Publication Number: WO 99/00520

(43) International Publication Date: 7 January 1999 (07.01.99)

(21) International Application Number: PCT/US98/13557

(22) International Filing Date: 29 June 1998 (29.06.98)

(30) Priority Data:
60/051,257 30 June 1997 (30.06.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/051,257 (CIP)
Filed on 30 June 1997 (30.06.97)

(71) Applicant (*for all designated States except US*): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, reresented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Bethesda, MD 20892 (US).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): KIRSCH, Ilan, R. [US/US]; 13321 Travilah Road, Potomac, MD 20854 (US).

(74) Agents: QUINE, Jonathan, A. et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: SPECTRAL CLONING–A NEW TECHNICAL APPROACH TO THE CLONING AND CHARACTERIZATION OF EVERY CHROMOSOMAL ABERRATION IN CANCER SAMPLES

(57) Abstract

Spectral cloning methods are provided for identifying and cloning all of the chromosomal abnormalities in a selected sample. Apparatus for plating and transferring up to 1,000,000 separate samples useful in the method are also provided. Kits for spectral cloning are provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

SPECTRAL CLONING-A NEW TECHNICAL APPROACH TO THE CLONING AND CHARACTERIZATION OF EVERY CHROMOSOMAL ABERRATION IN CANCER SAMPLES

FIELD OF THE INVENTION

The present invention relates to detecting the presence or absence of chromosomal abnormalities in specimens of interest, through the use of spectral cloning. In particular, the invention relates to the use of two or more labeled probes which provide a different detectable signal when co-localized at the site of a chromosomal abnormality, coupled with ready access to a viable phage or other vector containing a genomic insert containing the abnormality thus detected.

BACKGROUND OF THE INVENTION

A variety of chromosomal rearrangements (insertions, deletions, translocations, amplifications, deletions, and the like) are associated with abnormal cell growth and regulation, leading, *e.g.*, to tumorigenesis. The discovery of rearrangements associated with a particular tumor or other cancer cell provides the basis for clinical diagnosis of disease, prognosis, and selection of a therapeutic regimen. Furthermore, particular chromosomal abnormalities are often correlated with the misregulation or modification of particular genes important to cell growth, regulation and proliferation. The identification of these genes provides basic information on the processes of cell regulation and carcinogenesis and provides therapeutic targets for drug discovery efforts.

Several techniques are currently being used for detecting 8 chromosomal abnormalities, including optical mapping of chromosomes on surfaces (*see, e.g.,* Schwartz *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:5164-5168), Comparative Genomic Hybridization (CGH) (*see, e.g.,* Kallioniemi, *et al.* (1992) *Science* 258:818), spectral karyotyping (SKY) (*see, e.g.*, Garini, *et al. Bioimaging* 4:65-72 (1996)), and the like. Although many chromosomal abnormalities have been correlated with particular cell types (*e.g.*, particular tumors) using these methods, the prior art offers no way of expeditiously and

conveniently characterizing all of the chromosomal abnormalities associated with a particular cell type at the nucleotide level. Accordingly, it has not been possible to ensure that all relevant chromosomal abnormalities for a particular cell type have been elucidated, to determine the relative importance of particular abnormalities, or to consider globally the epigenetic effects of multiple chromosomal abnormalities.

The present invention overcomes the limitations of the prior art by, *inter alia*, providing methods and apparatus for delineating and cloning all of the rearrangements in a particular cell type or cell line.

SUMMARY OF THE INVENTION

This invention provides methods and related apparatus permitting the detection and characterization of all chromosomal abnormalities found in a biological sample such as a leukemia, carcinoma or sarcoma. In the methods, a genomic library is plated on a substrate (typically a matrix such as a solid substrate with up to about 1,000,000 wells, although agar based substrates are also contemplated). Nucleic acids isolated from the library are fixed to a support to create an array of nucleic acids. At least two probes having different detectable properties (*e.g.*, two different fluorescent or other colorimetric labels, or two different radioactive or two different co-reactive labels) are hybridized to the array. The probes correspond to different regions of the genome from which the genomic library was made, *e.g.*, YAC, BAC, MAC, P1, phage or plasmid clones of the genome. Nucleic acids in the array which bind to at least two (and optionally more) of these probes, where the probes are not derived from contiguous genomic clones, correspond to chromosomal abnormalities such as a chromosomal translocation, insertion, deletion or the like. Similarly, where probes derived from contiguous genomic regions do not both hybridize to a particular nucleic acid from an array, the nucleic acid can correspond to a chromosome rearrangement such as an insertion, translocation, amplification or the like.

Accordingly, in one class of embodiments, the present invention provides methods of detecting the presence or absence of a chromosomal abnormality in a target nucleic acid. In the methods, an array of nucleic acids is

provided, a first probe having a first spectrally detectable label is hybridized to the array of nucleic acids, a second probe having a second spectrally detectable label is hybridized to the array of nucleic acids and any target nucleic acid which is hybridized to each of the first and second probes to detect the presence of a chromosomal abnormality is identified.

In one group of these embodiments, the first and second probes are different colors, and when bound to the nucleic acid in close proximity, provide a spectral combination that yields a third color. Within this group of embodiments, a target nucleic acid is identified as being associated with the third color. The target nucleic acid can then be further characterized using cloning methods and sequencing. In particularly preferred embodiments, the first probe and the second probe are each independently a spectrally labeled YAC, a spectrally labeled BAC, a spectrally labeled MAC, a spectrally labeled P1 chromosome, a spectrally labeled plasmid, a spectrally labeled cosmid, a spectrally labeled phage, or the like.

In some embodiments, the array of nucleic acids comprises one or more DNA cloned from a chromosomal DNA, preferably obtained from a tumor cell.

Typically in the above methods, the array of nucleic acids will be provided by providing a culture of bacteria, infecting the culture of bacteria with a genomic phage library to provide phage infected bacterial cells, plating a sample of the phage infected bacteria on a substrate to provide an array of phage infected bacteria, culturing the phage infected bacteria to produce an amplified array of phage, making a corresponding copy of the array of phage and treating the amplified array of phage to produce an array of DNA, thereby providing the array of nucleic acids.

With regard to the apparatus aspects of the present methods, the substrate will preferably be made of nitrocellulose, glass, plastic or other polymer. The first and second probes are generally detected optically, optionally spectrophotometrically, in one preferred embodiment with a charge-coupled device, or with a phototube and photodiode. Radioactive signals are, however, optionally employed in a similar manner, with the ratio of different types of radioactive signals on different probes providing an indication of whether two or

more probes are bound to a particular target. It will be appreciated that scintillation or autoradiographic methods are employed when the labels are radioactive.

The nucleic acid arrays used in the present invention will, in one group of embodiments, comprise between about 100,000 and about 1,000,000 species of nucleic acids. In other embodiments, the array will comprise less than about 100,000 nucleic acids.

In preferred embodiments, the array of nucleic acids will be prehybridized to non-specific nucleic acids, preferably to COT 1 DNA, or to other blockers of repetitive DNA.

In a particularly preferred embodiment, the present invention provides a method of detecting a chromosomal abnormality comprising the steps of cloning DNA from a genomic sample into a phage library, infecting a bacterial culture with the phage library to provide infected bacterial cells, plating the infected bacterial cells onto a matrix under conditions permitting replication of phage in the phage library to make an array of phage, making a corresponding copy of the array of phage to produce a copy array, treating the phage in the array to produce an array of phage DNA, contacting the array of DNA with a first probe having a first color and a second probe having a second color, where the two probes are two differentially fluorescently labeled YAC or BAC DNA probes, and detecting the two probes.

For use in the methods of the invention it will be appreciated that the invention provides arrays of nucleic acids. In one aspect, the present invention provides an array of nucleic acids having a plurality of different nucleic acid species on a substrate and at least two probes comprising at least two different fluorescent labels hybridized to at least one nucleic acid species from the plurality of different nucleic acids. Preferably, the two fluorescent labels emit different wavelengths of light and the combination of the different wavelengths provides an optically distinct detectable color signal.

In yet another aspect, the present invention provides an apparatus comprising a solid substrate having at least 10,000 microwells at preselected locations on the solid substrate. Preferably, the apparatus will have at least 100,000 microwells, more preferably from about 100,000 to about 1,000,000

microwells. In preferred embodiments, a plurality of the microwells contain nucleic acids, phage, bacteria or combinations thereof. In other preferred embodiments, the microwells will have a volume of between about 0.1 nanoliters and 100 nanoliters, commonly between 0.1 and 50 nanoliters, more often between 0.1 and 10 nanoliters, and typically between about 0.1 and 1.0 nanoliters. In still other preferred embodiments, the apparatus further comprises an armature having a plurality of pins arranged to fit into a plurality of the microwells during operation of the apparatus for transfer of biological or chemical constituents to the microwells.

The present invention further provides kits for practicing the invention. Typical kits comprise any of the apparatus elements, library elements, or instructions for practicing the methods described above. Typical kits include any of the following: a container, a solid substrate having at least 10,000 microwells at preselected locations on the solid substrate, instructions for practicing the methods described herein, or the like.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 is a drawing of an apparatus of the invention.

Definitions

An "array" of nucleic acids is an ordered spatial arrangement of nucleic acids on a physical substrate. Row and column arrangements are preferred due to the relative simplicity in making and assessing such arrangements. However, the spatial arrangement can be essentially any form selected by the user. The nucleic acids in the array can be DNA, RNA, or an analogue thereof; however, DNA is generally preferred due to its relative ease of recombinant manipulation.

A "probe" is a detectable molecule which specifically hybridizes to a nucleic acid. Common probes useful in the present invention are labeled DNA plasmids, cosmids, phage, artificial chromosomes (YACs, BACs, MACs, P1 chromosomes *etc.*) and the like.

A "spectrally detectable" label is a label which emits one or more wavelengths of light upon exposure to an illumination source. For example, a

fluorescent label which emits light either spontaneously or after excitation by an illumination source is a spectrally detectable label.

A probe is "hybridized" to a nucleic acid when it is brought into proximity to the nucleic acid and allowed to associate based upon, *e.g.*, typical nucleic acid-nucleic acid interactions (hydrogen bonding, solvent exclusion, base stacking, *etc.*). Specific hybridization conditions are provided herein.

A "chromosomal abnormality" is a difference in the arrangement or composition of chromosomal DNA relative to a reference chromosome of the same type (*i.e.*, specific chromosome from a given species of organism). Selection of a "reference chromosome" is performed to elucidate what differences exist between a reference and a test sample. For example, chromosomal DNA from normal tissue serves as a reference when compared to chromosomal DNA from a cancerous tissue.

A "color" is a wavelength of light, or a ratio of two or more wavelengths of light. The wavelengths can be at, for example, visible, infrared, or ultraviolet frequencies.

A "microwell" is a depression in a solid substrate having a volume of less than 250 microliters, commonly less than 100 microliters, typically less than 10 microliters, often less than 1 microliter, commonly 500 nanoliters or less, often less than 100 nanoliters, optionally as little as 10 nanoliters, occasionally as little as 1 nanoliter, sometimes as little as 0.1 nanoliters. The well can have any shape, but will often be circular, triangular, square, rectangular or polygonal.

A "corresponding copy" of an original array is a copy array in which the spatial elements of the copy array can be related to the original array. Although it is convenient to make copies with the same spatial relationship as the original array, this is not necessary. As long as the relationship between the spatial elements of the original and the copy are known, the copy and the original can be corresponded.

DETAILED DISCUSSION OF THE INVENTION

Events which cause chromosomal abnormalities, such as insertions, deletions, and translocations, can lead to abnormal cell growth, and cancer. As cancer research has moved to the molecular level, it has become important to

determine the exact abnormality associated with individual types of cancers. Moreover, determination of the exact abnormality causing the cancer affecting individual patients has become important as cancer treatment has increasingly begun to move to the molecular and genetic level.

Current techniques for finding such abnormalities are neither quick nor convenient. Only gross abnormalities may be noted using standard "G-banding," or karyotyping using Giemsa staining to study the dark and light bands of the stained chromosomes. The practitioner cannot, however, necessarily identify an abnormally shaped chromosome or determine from which chromosome an abnormal section of another chromosome came. CGH can help identify whether a gain or a loss of material has occurred in a region, and fluorescent *in-situ* hybridization can identify only whether specific targets identified by specific probes are present or absent. Even recently developed techniques such as spectral karyotyping, or SKY, permit resolution only to a level of 1-10 megabases. Determination of the exact abnormality then requires positional cloning, a brute force technique which is labor intensive.

The present invention provides a quicker and more convenient means of elucidating chromosomal abnormalities. While the technique is discussed in greater detail below, it can be summarized as follows. Typically, cells from a sample of interest, (such as cancer cells from an established cell line or from a patient biopsy) are subjected to SKY, CGH, G-banding, or a combination of these or similar methods, to determine the presence of regions of gross or clear chromosomal abnormality. Cells of the sample are then used to create a genomic library, such as a phage library, which is then probed by YACs, BACs, or other probes (described in more detail below), selected to cover the region containing the abnormality, and differentially labeled. Once a particular phage or other vector containing the abnormal region is identified by detecting the combined signal of two or more probes, a viable phage or other vector containing the abnormal genomic insert is recovered from a previously created replica or corresponding array of the vector, isolated (if necessary),amplified, and sequenced. Thus, the technique permits an increase in resolution of the abnormality, for example, from the 1-10 million base level permitted by SKY to

the actual nucleotide sequence of the target region, without the need for arduous and labor intensive positional cloning.

Stated another way, phage or other genomic libraries are used to infect or transfect bacterial cells, which are plated, *e.g.*, on specialized substrates described herein, to provide an array of infected bacteria. The plated infected or transfected cells are cultured to provide an amplified clonal array of phage or of cells containing the genomic inserts, and a corresponding copy, sometimes termed a replica plate, is made. DNA (or RNA, if the phage is an RNA virus) is isolated from the clonal array, thereby providing an array of nucleic acids (typically DNA). The nucleic acid array is then hybridized to at least two labeled probes, which, when hybridized to the same nucleic acid fragment, provide a detectable marker distinct from either of the two labeled probes. For example, in one embodiment, one probe is fluorescently labeled and emits blue light, while the second probe, also fluorescently labeled, emits yellow light. When in close proximity, *i.e.*, when hybridized to the same nucleic acid, a green signal results. The presence (or absence) of this green signal (or other similar signal resulting from other color combinations) can be used as an indicator of the abnormal juxtaposition of two nucleic acid sequences, as occur, *e.g.*, when a chromosome is rearranged, or when a portion of the chromosome has an insertion, deletion, amplification, or the like. Target nucleic acids identified by this method can (by recovering viable cells or phage from the corresponding portion of the replica plate) be amplified, cloned, sequenced or the like, providing a method of identifying nucleic acid sequences in the region of a chromosomal abnormality.

The ability to detect, isolate, and recover all of the chromosomal abnormalities in a sample provides a fundamental new tool for laboratory use. For example, the ability to identify chromosomal aberrations provides a mechanism for discovering genes affected by aberration and, in combination with gene expression monitoring techniques, it is now possible to determine the connection between chromosomal aberrations and gene expression. Furthermore, with the methods and apparatus provided it is now possible to elucidate fundamental aspects of, *e.g.*, cancer biology, including whether chromosomal aberrations are cell lineage specific, what genomic chromosomal features can lead to chromosomal abnormalities, whether there is a distinct enzymatic mechanism

that mediates, *e.g.*, chromosomal rearrangements, detection of epigenetic effects and the like. Moreover, the ability to determine what abnormalities are associated with what particular cancers is of fundamental diagnostic and prognostic value to clinicians for treating and monitoring cancer. For example, sets of detection probes can be prepared for the detection of chromosomal abnormalities determined by the present methods which correlate to a particular cancer. By determining whether the abnormalities occur in a particular patient, it is possible to predict clinical outcomes and to adjust clinical treatments. Similarly, the methods of the invention can be used to determine all of the chromosomal breakpoints located in a particular cancer from a particular patient, thereby determining which associated genes are affected and providing a basis for designing a treatment regimen customized for that patient.

Libraries of Phage, Bacterial and Animal Culture, YAC and BAC construction, Nucleic Acid Sequencing

Methods of making genomic, mRNA, or expression libraries in bacteria, phage, and the like are well known. DNA sequencing, phage and bacterial culture conditions, useful in the methods of the present invention, as well as other related techniques are also well known, and are found, *e.g.,* in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, volume 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook, *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel, *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1997 Supplement) (Ausubel). A catalogue of Bacteria and Bacteriophages is provided, *e.g.,* by the ATCC, *e.g.,* The ATCC Catalogue of Bacteria and Bacteriophage (1992) Gherna *et al.* (eds) published by the ATCC. The above references and the references cited therein describe many methods of making recombinant RNA and DNA nucleic acids, including recombinant plasmids, recombinant lambda phage, cosmids, yeast artificial chromosomes (YACs), P1 artificial chromosomes, Bacterial Artificial Chromosomes (BACs), and the like, which are also generally known. A general introduction to YACs, BACs, PACs

and MACs as artificial chromosomes is described in Monaco and Larin (1994) *Trends Biotechnol* 12 (7): 280-286 and further information is found in Berger, Sambrook and Ausubel, *supra*.

In vitro amplification techniques suitable for amplifying sequences to provide a nucleic acid or for subsequent analysis, sequencing or subcloning are also known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA) are found in Berger, Sambrook, and Ausubel, as well as Mullis, *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis, *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3:81-94; Kwoh, *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli, *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell, *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren, *et al.*, *Science*, 241:1077-1080 (1988); Van Brunt, *Biotechnology*, 8:291-294 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer, *et al.*, *Gene*, 89:117 (1990), and Sooknanan and Malek, *Biotechnology*, 13:563-564 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace, *et al.*, U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids (up to 40 kb) are summarized in Cheng, *et al.*, *Nature*, 369:684-685 (1994) and the references therein. One of skill will appreciate that essentially any RNA can be converted into a double stranded DNA suitable for restriction digestion, PCR expansion and sequencing using reverse transcriptase and a polymerase. *See*, Ausubel, Sambrook, Innis, and Berger, *all supra*. Other general sources for any of these techniques are also well known to persons of skill.

Suitable methods for the detection of nucleic acids identified in the arrays of the invention include Southern analysis, PCR, northern analysis, *in situ* hybridization (including Fluorescent *In Situ* Hybridization ("FISH")), reverse chromosome painting, FISH on DAPI stained chromosomes, generation of Alphoid DNA probes for FISH using PCR, PRINS labeling of DNA, free chromatin mapping, spectral karyotyping and a variety of other techniques described, *e.g.*, in Tijssen (1993) Laboratory Techniques in biochemistry and molecular

biology--hybridization with nucleic acid probes parts I and II, Elsevier, New York, and, Choo (ed) (1994) Methods In Molecular Biology Volume 33- In Situ Hybridization Protocols Humana Press Inc., New Jersey (see also, other books in the Methods in Molecular Biology series). A variety of automated solid-phase detection techniques are also useful. For instance, very large scale immobilized polymer arrays (VLSIPS™), available from Affymetrix, Inc. in Santa Clara, CA are used for the detection of nucleic acids. *See*, Tijssen (*supra*), Fodor *et al.* (1991) *Science*, 251: 767- 777; Sheldon *et al.* (1993) *Clinical Chemistry* 39(4): 718-719, and Kozal *et al.* (1996) *Nature Medicine* 2(7): 753-759.

The source of biological material used to make a molecular library, in phage or in other vectors, varies depending on the intended application. Essentially any source of cells, including tissues, tumors, cell culture, or the like can be used as a source for making a molecular library for use in detecting and isolating all of the chromosomal abnormalities in the sample. For example, leukemias, sarcomas, carcinomas, and tumors (*e.g.*, colorectal tumors, lung tumors, prostate tumors, *etc.*) can all serve as the source for genomic DNA (or RNA) used to make a molecular library (including genomic and expression libraries). As discussed *supra*, generation of molecular libraries in bacteria, phage, or other vectors, cells or viruses is well known.

The culture of cells such as bacterial cells, animal cells, primary cells (cells isolated from a patient and optionally grown through 1-3 serial passages in culture), tumor cells and the like used in conjunction with the present invention, including cell lines and cultured cells from tissue or blood samples, is well known in the art. Freshney Culture of Animal Cells, a Manual of Basic Technique, third edition Wiley-Liss, New York (1994)) and the references cited therein provides a general guide to the culture of cells. See also, Kuchler, *et al.* (1977) *Biochemical Methods in Cell Culture and Virology*, Kuchler, R.J., Dowden, Hutchinson and Ross, Inc., and Inaba, *et al.*, *J. Exp. Med.*, 176:1693-1702 (1992). Additional information on cell culture is found in Ausubel, Sambrook and Berger, *supra*. Cell culture media are described in Atlas and Parks (eds) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, FL.

Most DNA sequencing is carried out by chain termination methods of DNA sequencing. The most popular chain termination methods of DNA sequencing are variants of the dideoxynucleotide mediated chain termination method of Sanger. *See*, Sanger *et al.* (1977) *Proc. Nat. Acad. Sci., USA* 74:5463-5467. Sequencing by hybridization to arrays of nucleic acids has also been described. *See,* U.S. Patent No. 5,202,231, to Drmanac *et al.* and, *e.g.*, Drmanac *et al.* (1989) *Genomics* 4:114-128. Methods of constructing and designing arrays for detection of single nucleotide alterations is also known in the art. *See,* Fodor *et al.* (1991) *Science*, 251: 767- 777; Sheldon *et al.* (1993) *Clinical Chemistry* 39(4): 718-719 and Kozal *et al.* (1996) *Nature Medicine* 2(7): 753-759. Small nucleic acids (oligonucleotides) used in the invention such as sequencing or PCR amplification primers and the like can be made recombinantly, but more typically they are made chemically according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), *Tetrahedron Letts.*, 22(20):1859-1862, *e.g.*, using an automated synthesizer, as described in Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.*, 12:6159-6168. Oligonucleotides can also be custom made and ordered from a variety of commercial sources known to persons of skill. Purification of oligonucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier (1983) *J. Chrom.* 255:137-149. The sequence of the synthetic oligonucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology* 65:499-560.

Labeling and Detecting Nucleic Acids

Identification of probe sequences for use in the methods of the invention is carried out by standard methods such as spectral karyotyping and CGH. In brief, patient samples or cell lines are selected based upon the information desired. Cell lines are one convenient starting material for cancer cells, including lung, breast and prostate cancer cell lines. A complete spectral karyotype and CGH analysis is performed on the selected sample. *See, e.g.,* Mitelman, *et al.* (1997) *Nature Genetics* April Issue; Schrock, *et al.* (1996)

Science 273: 494-497; Veldman, *et al.* (1997) *Nature Genetics* 15:406-410; Garini, *et al. Bioimaging* 4:65-72 (1996); and Kallioniemi, *et al.* (1992) *Science* 258:818. Using spectral karyotyping, FISH, and CGH methods, a complete karyotype of the sample is made. Molecular clones (*e.g.*, from YAC, BAC or other libraries) corresponding to regions of potential chromosomal abnormalities are selected from available libraries. Optionally, EST or Sequence Tagged Site ("STS") maps are used to isolate corresponding YAC, BAC or other probes from an appropriate genomic library by probing the library with a probe corresponding to a known EST or STS.

YAC, BAC, or other clones which are selected are labeled and used to probe the arrays of the invention. A label can be any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include spectral labels such as fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, Cy5.5, spectrum green, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.),* enzymes (*e.g.*, horse-radish peroxidase, alkaline phosphatase *etc.*) spectral colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*), beads. The label may be coupled directly or indirectly to a component of the assay (*e.g.*, a YAC or a BAC) according to methods well known in the art. Alternatively, the probe may be labeled with a moiety such as biotin, which can then be detected by a ligand, such as strepavidin, which is fluorescently labeled. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions. In general, a detector which monitors a probe- target nucleic acid hybridization is adapted to the particular label which is used. Typical detectors include spectrophotometers, phototubes and photodiodes, microscopes, scintillation counters, cameras, film and the like, as well as combinations thereof. Examples of suitable detectors are widely available from a variety of commercial sources known to persons of skill. Commonly, an optical image of a substrate comprising a nucleic acid array with particular set of probes bound to the array is digitized for subsequent computer analysis.

Fluorescent labels are particularly preferred labels. Preferred labels are typically characterized by one or more of the following: high sensitivity, high stability, low background, low environmental sensitivity and high specificity in labeling. Fluorescent moieties, which are incorporated into the labels of the invention, are generally are known, including Texas red, 1- and 2-aminonaphthalene, p,p'- diaminostilbenes, pyrenes, quaternary phenanthridine salts, 9-aminoacridines, p,p'-diaminobenzophenone imines, anthracenes, oxacarbocyanine, merocyanine, 3-aminoequilenin, perylene, bis-benzoxazole, bis-p-oxazolyl benzene, 1,2-benzophenazin, retinol, bis-3-aminopyridinium salts, hellebrigenin, tetracycline, sterophenol, benzimidazolylphenylamine, 2-oxo-3-chromen, indole, xanthen, 7-hydroxycoumarin, phenoxazine, calicylate, strophanthidin, porphyrins, triarylmethanes and flavin. Individual fluorescent compounds which have functionalities for linking to an element desirably detected in an apparatus or assay of the invention, or which can be modified to incorporate such functionalities include, *e.g.*, dansyl chloride; fluoresceins such as 3,6-dihydroxy-9-phenylxanthydrol; rhodamineisothiocyanate; N-phenyl 1-amino-8-sulfonatonaphthalene; N-phenyl 2-amino-6-sulfonatonaphthalene; 4-acetamido-4-isothiocyanato-stilbene-2,2'-disulfonic acid; pyrene-3-sulfonic acid; 2-toluidinonaphthalene-6-sulfonate; N-phenyl-N-methyl-2-aminoaphthalene-6-sulfonate; ethidium bromide; stebrine; auromine-0,2-(9'-anthroyl)palmitate; dansyl phosphatidylethanolamine; N,N'-dioctadecyl oxacarbocyanine: N,N'-dihexyl oxacarbocyanine; merocyanine, 4-(3'-pyrenyl)stearate; d-3-aminodesoxy-equilenin; 12-(9'-anthroyl)stearate; 2-methylanthracene; 9-vinylanthracene; 2,2'(vinylene-p-phenylene)bisbenzoxazole; p-bis(2-(4-methyl-5-phenyl-oxazolyl))benzene; 6-dimethylamino-1,2-benzophenazin; retinol; bis(3'-aminopyridinium) 1,10-decandiyl diiodide; sulfonaphthylhydrazone of hellibrienin; chlorotetracycline; N-(7-dimethylamino-4-methyl-2-oxo-3-chromenyl)maleimide; N-(p-(2- benzimidazolyl)-phenyl)maleimide; N-(4-fluoranthyl)maleimide; bis(homovanillic acid); resazarin; 4-chloro-7-nitro-2,1,3- benzooxadiazole; merocyanine 540; resorufin; rose bengal; and 2,4-diphenyl-3(2H)-furanone. Many fluorescent tags are commercially available from SIGMA chemical company (Saint Louis, MO), Molecular Probes,

R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersberg, MD), Fluka Chemica- Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), and Applied Biosystems (Foster City, CA) as well as other commercial sources known to one of skill.

In one embodiment, nucleic acids are labeled by culturing recombinant cells which encode the nucleic acid in a medium which incorporates fluorescent nucleotide analogues in the growth medium, resulting in the production of fluorescently labeled nucleic acids. Similarly, nucleic acids are synthesized *in vitro* using a primer and a DNA polymerase such as *taq*, or by "nick translation." For example, Hawkins *et al.* U.S. Pat. No. 5,525,711 describes pteridine nucleotide analogs for use in fluorescent DNA probes.

Fluorescent labels are one preferred class of detectable labels, in part because by irradiating a fluorescent label with light, one can obtain a plurality of emissions. Thus, a single label can provide for a plurality of measurable events. Detectable signal may also be provided by chemiluminescent and bioluminescent sources. Chemiluminescent sources include a compound which becomes electronically excited by a chemical reaction and may then emit light which serves as the detectible signal or donates energy to a fluorescent acceptor. A diverse number of families of compounds have been found to provide chemiluminescence under a variety or conditions. One family of compounds is 2,3-dihydro-1,4-phthalazinedione. The most popular compound is luminol, which is a 5-amino compound. Other members of the family include the 5-amino-6,7,8-trimethoxy- and the dimethylamino[ca]benz analog. These compounds can be made to luminesce with alkaline hydrogen peroxide or calcium hypochlorite and base. Another family of compounds is the 2,4,5-triphenylimidazoles, with lophine as the common name for the parent product. Chemiluminescent analogs include para-dimethylamino and -methoxy substituents. Chemiluminescence may also be obtained with oxalates, usually oxalyl active esters, *e.g.*, p-nitrophenyl and a peroxide, *e.g.*, hydrogen peroxide, under basic conditions. Other useful chemiluminescent compounds are also known and available, including -N-alkyl acridinum esters (with H_2O_2 under basic

conditions) and dioxetanes. Alternatively, luciferins may be used in conjunction with luciferase or lucigenins to provide bioluminescence.

The label is coupled directly or indirectly to a molecule to be detected (a product, substrate, enzyme, or the like) according to methods well known in the art. As indicated above, a wide variety of labels are used, with the choice of label depending on the sensitivity required, ease of conjugation of the compound, stability requirements, available instrumentation, and disposal provisions. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to a nucleic acid such as a YAC or BAC. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with labeled, anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody. Labels can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore or chromophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography.

Desirably, fluorescent labels absorb light above about 300 nm, preferably about 350 nm, and more preferably above about 400 nm, usually emitting at wavelengths greater than about 10 nm higher than the wavelength of the light absorbed. It should be noted that the absorption and emission characteristics of the bound label may differ from the unbound label. Therefore, when referring to the various wavelength ranges and characteristics of the labels, it

is intended to indicate the labels as employed and not the label which is unconjugated and characterized in an arbitrary solvent.

Fluorescent or similar labels and detection techniques, particularly microscopy and spectroscopy are preferred. Enzymatic labels are also detected by providing appropriate substrates for the enzyme and detecting the resulting reaction product. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence, *e.g.*, by microscopy, visual inspection, via photographic film, by the use of electronic detectors such as digital cameras, charge coupled devices (CCDs) or photomultipliers and phototubes, and the like. Arbitrary colors are assigned to particular ratios of wavelengths of emitted light. For example, two probes, each of which comprise a different fluorophore, when bound to nucleic acids in a single well on a substrate of the invention, will emit light at two different wavelengths. The pixel by pixel spectrum of two chromophores is arbitrarily assigned to be a particular color. In some cases, *e.g.*, where the first fluorophore emits a "blue" light and the second fluorophore emits a "yellow" light, the effect to the observer will be that a "green" signal will be observed. That is, two emission spectra will combine to yield a third spectra. Even when the fluorophores emit a non-visible wavelength of light (for example, in the infrared or ultraviolet portions of the spectrum), a color can be assigned to a ratio between any two (or more, *e.g.*, where more than two probes are used in an assay) wavelengths of light.

In fluorescence-based microscopy, light is typically directed into the specimen field at an angle which minimizes the amount of illumination light that enters the observation optics, for example it passes through the optics in a direction opposite to the light path of the observer. Use of filters to remove remaining stray illumination and scattered light makes the field appear dark to an observer. Excitation of fluorophores in the fluorescent sample give off secondary light, making the specimen appear bright and/or colored (brightness and color optionally occur outside of the visual range, in which case they are detected, *e.g.*, artificially). Fluorescence-based microscopy is well known, *e.g.*, for the detection and quantification of inorganic, organic and biological polymers. Fluorescence is analyzed in clinical settings to obtain measurements in connection with immunology, toxicology, microbiology, drug screening, clinical chemistry,

histopathology, and the like. Fluorescence is analyzed in many contexts to study enzymes, amino acids, carcinogens, and a wide variety of other chemical compounds. Nucleic acids such as DNA and RNA, proteins, chromosomes and other macromolecular structures are all visualized by fluorescence-based microscopy and the techniques suitable to these applications are suitable here. Similarly, arrays of biological polymers are monitored by fluorescence-based microscopy for nucleic acid sequencing by hybridization, detection of genetic polymorphisms, drug screening and many other uses. For instance, comparative genomic hybridization (CGH) is a well-known approach for identifying the presence and localization of amplified or deleted sequences in a genome compared to a reference genome. *See*, Kallioniemi, *et al.* (1992) *Science* 258:818 and Pinkel *et al.* PCT/US95/16155 (WO 96/17958).

Optical images viewed (and, optionally, recorded) by a camera or other recording device (*e.g.*, a photodiode and data storage device) are optionally further processed in any of the embodiments herein, *e.g.*, by digitizing the image and storing and analyzing the image on a computer. A variety of commercially available peripheral equipment and software is available for digitizing, storing and analyzing a digitized video or digitized optical image, *e.g.*, using PC (Intel x86 or pentium chip- compatible DOS™, OS2™ WINDOWS™, WINDOWS NT™ or WINDOWS95™ based machines), MACINTOSH™, or UNIX based (*e.g.*, SUN™ work station) computers.

One conventional system carries light from the specimen field to a cooled charge-coupled device (CCD) camera, in common use in the art. A CCD camera includes an array of picture elements (pixels). The light from the specimen is imaged on the CCD. Particular pixels corresponding to regions of the specimen (*e.g.*, individual hybridization sites on an array of biological polymers) are sampled to obtain light intensity readings for each position. Multiple pixels are digitized and processed in parallel by computer to increase the speed of analysis.

The preferred size of the substrate upon which arrays are formed in the present invention is a function of image analysis. In certain embodiments, it is preferable to image the entire array simultaneously. In these embodiments, it is desirable to make the array small (typically less than 100 cm^2, often less than 10

cm^2, sometimes less than about 1 cm^2) to permit simultaneous imaging of the array. Simultaneous analysis of the entire array permits high throughput screening by speeding image analysis. Thus, it is desirable to use a substrate or matrix which can be imaged by the detection optics. However, larger arrays are also useful where an image of the entire array is constructed by gradually scanning a large array, *e.g.*, by scanning the array pixel by pixel, digitizing the image, and analyzing the image using a computer.

One of skill will immediately recognize that any, or all of the components of the apparatus and detection means for monitoring a signal on a substrate in the assays of the invention are optionally manufactured in separable modular units, and assembled to form an apparatus of the invention. In particular, a wide variety of substrates having different channels, wells and the like are typically manufactured to fit interchangeably into a substrate holder, so that a single apparatus can accommodate, or include, many different substrates adapted to control a particular reaction. Multiple pin units are optionally substituted on one or more armature units in the apparatus of the invention. Similarly, computers for recording and assessing signals on a substrate, detectors armature and pin units and substrate holders are optionally manufactured in a single unit, or in separate modules which are assembled to form an apparatus for manipulating and monitoring a substrate. In particular, a computer does not have to be physically associated with the rest of the apparatus to be "operably linked" to the apparatus. A computer is operably linked when data is delivered from other components of the apparatus to the computer. One of skill will recognize that operable linkage can easily be achieved using either electrically conductive cable coupled directly to the computer (*e.g.*, parallel, serial or modem cables), or using data recorders which store data to computer readable media (typically magnetic or optical storage media such as computer disks and diskettes, CDs, magnetic tapes, but also optionally including physical media such as punch cards, vinyl media or the like). Most typically, data is delivered to a computer in digital form from a detector such as a CCD, phototube or the like.

The present invention is further illustrated by consideration of Fig. 1. Solid support **101** having, *e.g.*, from about 10,000 to about 1,000,000 "pits"

or wells **104** is operationally mounted to or in proximity to armature **102** supporting pin array **103** having pins **105** arranged to fit into wells **104**.

Loading Solid Substrates

The solid substrates used in preparing arrays of nucleic acids can be any support which is compatible with the nucleic acids which are plated onto the support. Accordingly, the support can be biological, nonbiological, organic, inorganic, or a combination of any of these. The solid support will typically exist in a rectangular form although other shapes (*e.g.*, square, circular, oval, triangular, *etc.*) are also contemplated within the present invention. The solid support is preferably substantially flat but may take on alternative surface configurations. For example, the solid support may contain raised or depressed regions on which the nucleic acids are fixed. In particularly preferred embodiments, the solid support will be a rectangular surface having a plurality of "wells" into which the nucleic acids are plated. In some embodiments, the solid support will be chosen to provide appropriate light-absorbing characteristics. For example, the support may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SiN_4, modified silicon, or any one of a variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidendifluoride, polystyrene, polycarbonate, polypropylene, or combinations thereof. Other suitable solid support materials will be readily apparent to those of skill in the art (*e.g.*, divinylbenzene-styrene, polyacrylamides, acrylic acid grafted polypropylene, and the like).

As noted above, the solid support will typically have a plurality of "wells" or regions within which the nucleic acids can be plated. In one group of embodiments, the solid support will have from about 100,000 to about 1,000,000 wells. In other embodiments, the solid support will have fewer than about 100,000 wells. Solid supports having a plurality of wells can be prepared used any of a variety of art-recognized techniques, including laser etching, dip masking or microdrilling. Preferably, each of the wells is at a preselected location on the solid support (*e.g.*, a uniquely identifiable position characterized by x and y coordinates).

In another group of embodiments, the wells will each have a capacity of from about 0.1 nanoliters to about 100 nanoliters, commonly between about 0.1 and about 10 nanoliters and typically between about 0.1 and about 1.0 nanoliters.

In one group of embodiments, nucleic acids, phage, or other biological materials are placed into each well on a support using micropins. The pins, like the wells, are arranged at predetermined positions on an armature. The pins are preferably made of inert materials such as, for example, polyacrylamides, controlled-pore glass, polycarbonate and the like. The pins are preferably arranged at positions on the armature (*see* Fig. 1) which correspond to positions on each of the nucleic acid array support and the "duplicate" or corresponding array support. For example, a first pin $P_{1,1}$ is located at a position corresponding to x_1,y_1, a second pin $P_{1,2}$ is located at a position corresponding to x_1,y_2, a third pin $P_{2,1}$ is located at a position corresponding to x_2,y_1, *etc.* In this manner, each pin can be used to deposit nucleic acids in predetermined wells in both a first array and a second corresponding or duplicate array. Although it is often convenient to make duplicate copies of an array in the same configuration as an original, this is not a requirement. As long as the spatial arrangement of elements on one substrate can be corresponded to the elements on a second substrate a corresponding copy can be made. For example, the spatial position on one substrate can arbitrarily be corresponded to the spatial position on a second substrate.

Substrates of the invention, typically having at least 5,000 wells, often at least 10,000, generally at least 50,000, typically at least 100,000, and up to at least 1,000,000 wells are loaded with culture materials, generally including bacteria, vector particles (*e.g.*, phage library materials), and the like. The substrates are typically loaded by immersion in solution, by pipetting materials onto the surface of the substrate, or directly into wells, or using pins as described. Immersion, pipetting, pin transfer, or similar loading techniques are performed manually, or using an automated system such as a robotic pipettor. For example, the Zymate XP (Zymark Corporation; Hopkinton, MA) automated robot using Microlab 2200 (Hamilton; Reno, NV) pipetting station can be used to transfer cell samples to a 96-well microtiter plate. A similar arrangement is optionally used to

load the wells of the invention, *e.g.*, using a robotic pin armature which mates with a selected substrate. Immersion methods are also preferred methods, due to the general simplicity in loading many wells simultaneously.

Producing Arrays; Hybridization of Materials to the Arrays

As described, phage or other library components (plasmid-containing bacteria, viruses, *etc.*) are plated onto an original substrate and cultured under conditions which permit replication. (For convenience, the discussion will assume the library component used is a phage, although other components could of course be used.) A corresponding copy of the plated replicated phage is produced, *e.g.*, by pin transfer to a second substrate. To make a nucleic acid array, the DNA from the original substrate can be transferred to a second corresponding substrate for analysis (it will be appreciated that a phage lysate comprises phage and unencapsulated phage DNA) by pin transfer. Alternatively, the phage on the original or duplicate substrate can be lysed by incubation with NaOH, EDTA plus heat, or any other common method for phage lysis. In chemical methods, desired chemicals are micropipetted onto the substrate, or by pin transfer of the chemicals. The solution in the wells containing the lysed phage is then neutralized, and the DNA denatured (*e.g.*, by heat or by common chemical DNA denaturation methods), again with appropriate chemical elements being introduced by micropipetting or pin transfer. The resulting denatured DNA is fixed to the substrate using appropriate methods, depending on the nature of the substrate. Common DNA fixing methods include UV cross linking, baking, chemical coupling and the like. The resulting fixed DNA is arranged in an array corresponding to the location of wells on the substrate.

Probes comprising YACs, BACs, P1 plasmids, plasmids, MACs and the like are typically hybridized to the resulting array after the array is treated with a blocking reagent to reduce non-specific hybridization of the probes. COT 1 DNA is a preferred blocking agent, because it tends to reduce binding of probes to common repetitive sequences. However, other common hybridization competitors such as calf thymus DNA, salmon sperm DNA, poly d(IC) or the like can also be used.

Probe hybridization conditions vary depending on the particular probe. It is expected that one of skill is fully knowledgeable in the hybridization of nucleic acids. Two single-stranded nucleic acids "hybridize" when they form a double-stranded duplex. The region of double-strandedness can include the full-length of one or both of the single-stranded nucleic acids, or all of one single stranded nucleic acid and a subsequence of the other single stranded nucleic acid, or the region of double-strandedness can include a subsequence of each nucleic acid. An overview to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York.

"Stringent conditions" in the context of nucleic acid hybridization are sequence dependent and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993), *id.* Generally, stringent conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Highly stringent conditions are selected to be equal to the T_m point for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues is 50% formalin with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of stringent wash conditions is a .2x SSC wash at 65°C for 15 minutes (*see,* Sambrook, *supra* for a description of SSC buffer). Often the high stringency wash is preceded by a low stringency wash to remove background probe signal. An example low stringency wash is 2x SSC at 40°C for 15 minutes for a probe with at least about 100 complementary nucleic acids. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

Nucleic acids in an array which are identified as binding to multiple probes from different chromosomal regions correspond to chromosomal

abnormalities. Similarly, nucleic acids in the array which bind to multiple probes in an abnormal fashion also represent nucleic acids of interest. Nucleic acids of interest are typically assessed by any of a variety of techniques such as PCR amplification, cloning and sequencing to determine the structure or sequence of the target nucleic acid. It will be appreciated that the corresponding copy of an array is especially useful for cloning or amplifying a nucleic acid of interest. Phage located on the corresponding copy are cloned (and if necessary re-screened by limiting dilution to isolate a phage encoding the nucleic acid of interest), subcloned, sequenced, PCR amplified or the like, depending on the intended application using standard molecular cloning techniques, *e.g.*, as described in Sambrook, Innis, Berger, and Ausubel (*all supra*).

Sequence information from the identified nucleic acid is used, *e.g.*, to construct databases of the chromosomal abnormalities which correspond to a given biological source material (particular tumor type, *etc.*, used to make the phage library which is screened). This database information provides a basic tool for clinicians and researchers for diagnostic, prognostic and basic research applications.

Adjusting the Number of Wells

One advantage offered by spectral cloning conducted on large microwell arrays is that it permits seeding the phage or other vector at very low density. Using a million wells, for example, phage vectors containing a mammalian genomic library (which need about 800,000 genomic fragments to reach a 99% probability that every sequence of the genome is represented in at least one vector, *see, e.g.*, B. Lewin, *Genes IV*, (Cell Press, Cambridge, MA and Oxford Press, New York, NY, 1990), at 460-461), can be seeded at an average density of 1 phage per well or less. When the phage are grown in their wells, they are therefore available for identification through spectral cloning and further sequencing or the like without first needing additional steps to select for the phage of interest. While this is most conveniently done using substrates with a large number of wells, it can also be performed, at some cost in convenience in screening and handling, using arrays containing a relatively small number of wells per substrate. For example, approximately 10,000 standard laboratory 96 well

microtiter plates ("96-well plates" or "plates") can be seeded with phage containing a human genome to obtain an average density of 1 phage per well.

In some applications, however, it will not be necessary to use such a large number of wells to achieve low density seeding of the phage or other vector. For example, not all genomes are of the same complexity as mammalian genomes, and for less complex genomes, correspondingly fewer cloned fragments are necessary to achieve high levels of probability that all the segments of the genome have been packaged into at least one of the vectors that comprise the library. To illustrate, for *E. coli*, only some 1500 cloned fragments are needed to reach a probability level of 99% that every sequence of the genome is represented, while the necessary size increases to 4600 for yeast and 48,000 with *Drosophila*. *Id.* As a second example, only a portion of a genome, such as a particular chromosome, may be of interest. Here, once again, a correspondingly smaller array will permit seeding of the phage or other vector at low density. Moreover, in some applications, the practitioner may not need as high a level of probability that every sequence of the genome is represented. In these instances, too, a smaller number of wells can be used overall to conduct the technique.

Spectral cloning does not, however, require the use of low density seeding of phage or other vectors. When using 96-well plates, it will accordingly generally be more convenient to use a smaller number of plates, seeded at a higher density. Higher densities of phage (or other vectors) can result in some savings in time, cost, and reagent use. On the other hand, once a well with a phage (or other vector) containing a genomic insert of interest is probed and identified, it will then be necessary to isolate the phage (or other vector) with the insert of interest (which can be done by standard methods known in the art, such as plaque purification).

The convenience of seeding wells at higher density should therefore be balanced against the loss of convenience in subsequently isolating the vector of interest. It will be appreciated that, as the number of phage or other vector seeded in a well increases, there will be a corresponding decrease in that well in the relative percentage of the phage or other vector containing the chromosomal segment of interest. As the density of the phage or other vector seeded per well increases, it will therefore be necessary to probe a larger aliquot of culture from

each well to identify wells containing phage or other vectors of interest. The size of the necessary aliquot can be readily calculated by the practitioner given any particular density of seeding. If automated probing and optical imaging of the hybridization array is contemplated, it will be preferred to use small aliquots for screening so that more aliquots can be screened by the device at a time. For uses where automated screening is contemplated, therefore, it will be preferred to use seeding densities of phage or other vectors below 25,000 per well, more preferred to use seeding densities below about 10,000 per well, still more preferred to use seeding densities at or below about 1,000 per well, and even more preferred to use seeding densities below about 500 per well. Most preferred are seeding densities between about 2 and about 100 phage or other vectors per well.

Although 96-well plates have the advantage of ready availability, one can of course also use other commercially available plates with different numbers of wells. Preferably, the plates will have a higher number of wells, such as the 1536-well plate available from Corning Costar Corp. (Acton, MA). The number of plates or other substrates needed to conduct the technique with respect to any particular genome or portion of a genome will, of course, vary in roughly inverse proportion to the number of wells or microwells on the plate or substrate used and the density of seeding desired.

The practitioner can also vary the vector used. Different vectors carry genomic inserts of different average sizes. While the discussion above has been focused on the use of phage, vectors such as cosmids and plasmids, which carry genomic inserts larger than can be carried by phage, can also be used. As the size of the genomic insert carried by the vector increases, the smaller are the number of the vectors required to carry the entire genome of interest, and the smaller the number of wells which must be used to seed the vectors at any desired density. The practitioner can easily calculate the number of wells needed to account for these variations.

Use to Detect Fusion RNAs or cDNAs

Some cancers and other conditions result from abnormal gene products, such as fusion proteins caused by fusion RNAs due to translocations. One of the first cancers associated with a chromosomal aberration was a form of

chronic myelogenous leukemia caused by the so-called "Philadelphia chromosome," resulting from a translocation of chromosomes 9 and 22, which normally express gene products termed *bcr* and *abl*. The translocation results in a fusion product *bcr/abl*, which is part *bcr* and part *abl*. Similarly, anaplastic large cell non-Hodgkin's lymphoma results from a translocation of chromosomes 2 and 5, resulting in a *NPM/ALK* fusion. Other fusion genes, such as a series associated with childhood sarcomas, are also known. The method of the invention can also be used to detect these abnormalities.

In this use, a practitioner suspecting a chromosomal abnormality (perhaps detected through SKY, CGH, or the like) first extracts RNA from a patient sample (or other source) by any of a number of standard methods known in the art (such as those taught in Chapter 7 of Sambrook, *supra*). The RNA is then hybridized with, for example, a labeled YAC, BAC, plasmid, or cDNA specific for the affected area of a first chromosome, and a second, differently labeled YAC, BAC, plasmid, or cDNA specific for the affected area of a second chromosome, and the sample is observed to detect the presence or absence of a third color arising from the co-localization of the two labeled probes on the same portion of the RNA.

Alternatively, a cDNA library is created from the patient sample (or other source) by standard methods (such as those taught in Chapter 8 of Sambrook, *supra)*, the cDNA is denatured, and then hybridized with, for example, a labeled YAC, BAC, plasmid, or cDNA specific for the affected area of a first chromosome, and a second, differently labeled YAC, BAC, plasmid, or cDNA specific for the affected area of a second chromosome, and the sample is observed to detect the presence or absence of a third color arising from the co-localization of the two labeled probes on the same portion of the cDNA.

EXAMPLES

The following examples are offered to illustrate, but not to limit, the claimed invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the

appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

Example 1: Spectral Cloning

A glass slide is micro-etched with 100,000-1,000,000 "pits" (wells). Into each of these pits is seeded *E. coli* bacteria, followed by 0-3 (average 1-2) of the bacteriophage from the genomic library made from a patient sample(s) or cell line(s). The slide is incubated to allow the phage to infect and lyse the bacteria in each pit. A replica of the resulting phage lysate plate is made and stored. On the original plate, DNA is made accessible *in situ* from the phage in each pit. The DNA in each pit is denatured and fixed to the sides of its respective pit. A prehybridization step is carried out with Cot-I DNA to block the repetitive sequences on the phage clones. Following this prehybridization, high stringency hybridization is carried out using a mixture of fluorescently labelled YAC or BAC clones that are derived from regions covering the breakpoints or other chromosomal abnormality identified by, for example, SKY or CGH. For example, if a t(2;4) translocation is noted, YAC clones covering the relevant region of chromosome 2 might be labelled yellow and YAC clones covering the analogous region of chromosome 4 might be labelled blue. This mixture would be applied to the slide. Following hybridization and "washing" the slide is subjected to image analysis. Occasional blue and yellow pits are identified, representing hybridization of one or the other YACs to their homologous internal sequences. Less frequently a "green" pit is seen. This green pit represents a phage clone on which exist sequences hybridizing to both one of the chromosome 2 YACs and one of the chromosome 4 YACs. This most likely represents a phage clone that carries the t(2;4) breakpoint or its reciprocal (the chance that a phage from that specific regions of chromosome 2 would have, randomly, landed in the same pit with a phage from the analogous region of chromosome 4 is low). Having identified the relevant pit, the corresponding living phage would be recovered from the replica plate, grown, cloned, and, following DNA preparation, mapped and sequenced to yield the breakpoint sequence. With this sequence in hand, reference back to a genome sequence, EST database, *etc.* is performed to place the breakpoint more accurately and identify candidate genes affected by the breakpoint

(in some cases it is anticipated that the rearrangement itself directly disrupts a transcript unit).

Example 2: Agarose Substrate Method

In this version of "spectral cloning" the relevant phage library is plated on bacterial lawns on multiple agarose plates or trays. Hybridizations are carried out on nitrocellulose or Nytran overlays. The probes are either fluorescent or possibly chemical in which, for example, a reactive group A would label YACs from chromosome 2 (in the above example of a t(2;4) translocation) and reactive group B would label YACs from chromosome 4. Only within a plaque containing sequences from both chromosomes 2 and 4 would reactive group A interact with reactive group B yielding a colorimetrically detectable spot on the filter. The steps following this identification of the rearranged clone are similar to those described above.

Example 3: Producing High Titer Phage Lysates in Microtiter Wells

This Example shows the production of high-titer phage lysates in microtiter wells.

A fresh culture of a suitable bacterium, such as *E. coli* XL-1 Blue (P2) (Stratagene, inc., La Jolla, CA) is started in YTM ("Yeast tryptone medium," *see* Sambrook, *supra*). Approximately 100 ml of culture are grown at 37°C to an O.D. of approximately 1.0 (which is equivalent to roughly 8 x 10^8 cells/ml). The culture of cells is divided into two or more tubes and centrifuged in a refrigerated tabletop centrifuge at 2500 rpm for 10 minutes. Half of the cells (*i.e.*, one tube) are resuspended in 10 mM $MgSO_4$ at a concentration of 8 x 10^8 cells/ml. The bacteria are mixed with a Bacteriophage λ-FIX (Stratagene, Inc., La Jolla, CA) phage library at a m.o.i. of 1 phage to 80 bacteria (for example, 8x 10^8 bacteria/ml are mixed with 10^7 phage/ml). The phage are permitted to adsorb for 20 minutes at 37°C. During the adsorption, the other half of the pelleted culture is resuspended in warm LB ("Luria-Bertani," *see*, Sambrook, *supra*) broth at 10^8 bacteria/ml, and 140 μl aliquots of the culture are placed into designated wells of a 96-well microtiter plate.

Following the adsorption step, the adsorption mix is diluted 1:1000 in warm LB broth, and 10 μls of diluted adsorption mix (containing about 100 bacteriophage) are aliquoted into designated wells already containing aliquots of the bacterial culture. The microtiter plate is incubated at 37°C for 8 hours, under shaking conditions, which permits repeated cycles of phage growth and bacterial lysis.

After the incubation, 5 μl of $CHCl_3$ is added to each well. The titer of phage in each well should range between 10^9 and 5 x 10^9 /ml. The microtiter plate is covered with a plastic plate cover and sealed until further processing is performed. The plate can be stored at 4°C for up to a month.

Example 4: Screening of a Human Genomic Library Using Microtiter Plates

This Example illustrates use of spectral cloning using 96-well microtiter plates.

To screen a human genomic library, one million phage from a selected library (approximately 5 genomes equivalent; typically more than one genome equivalent is screened to reach a high level of probability that everything which is capable of being represented in a library made with the particular restriction enzyme used is subjected to the screen) can be screened by seeding 10-100 phage per well in 100 96-well microtiter plates. The phage are then grown to a titer of 10^9/ml, as shown in Example 3, above. Aliquots are then drawn from each well and each aliquot is placed on a position on a replica plate designated to correspond to the spot on the original plate. Conveniently, the amount aliquoted is about 1 nanoliter, and contains about 1000 phage. In this Example, hybridization and detection of abnormalities is then conducted on the small aliquot in the duplicate array. If the initial seeding of a particular well contained 1 phage of interest (that is, containing a particular chromosomal abnormality of interest from the genomic library), there should be about 10 phage of interest in the spot hybridized available for detection by probes for that chromosomal abnormality.

WHAT IS CLAIMED IS:

1. A method of detecting the presence or absence of a chromosomal abnormality in a target nucleic acid, comprising:

providing an array of nucleic acids;

hybridizing a first probe having a first spectrally detectable label to the array of nucleic acids;

hybridizing a second probe having a second spectrally detectable label to the array of nucleic acids; and

identifying any target nucleic acid which is hybridized to each of said first and said second probes to detect the presence of a chromosomal abnormality.

2. The method of claim 1, wherein said first detectable label is a first color, said second spectrally detectable label is a second color and wherein the first and second labels, when in proximity, provide a third color.

3. The method of claim 2, further comprising identifying a target nucleic acid, detectable by the third color.

4. The method of claim 3, further comprising amplifying said target nucleic acid.

5. The method of claim 4, further comprising sequencing at least a portion of said target nucleic acid.

6. The method of claim 3, further comprising cloning said target nucleic acid.

7. The method of claim 1, wherein the first probe and the second probe are selected from the group of probes consisting of a spectrally labeled YAC, a spectrally labeled BAC, a spectrally labeled P1 artificial chromosome, a spectrally labeled MAC, a spectrally labeled plasmid, a spectrally labeled phage, and a spectrally labeled cosmid.

8. The method of claim 1, wherein the array of nucleic acids comprises one or more DNA cloned from a chromosomal DNA.

9. The method of claim 1, wherein the chromosomal DNA is obtained from a tumor cell.

10. The method of claim 1, further comprising the steps of:

providing a culture of bacteria;

transfecting the culture of bacteria with a genomic library to provide transfected bacterial cells;

plating a sample of the transfected bacteria on a substrate to provide an array of transfected bacteria;

making a corresponding copy of the array of transfected bacteria; and,

treating the array of transfected bacteria to produce an array of DNA, thereby providing the array of nucleic acids.

11. The method of claim 10, further comprising the step of recovering viable transfected bacteria from the corresponding copy.

12. The method of claim 1, further comprising the steps of:

providing a culture of bacteria;

infecting the culture of bacteria with a genomic phage library to provide phage infected bacterial cells;

plating a sample of the phage infected bacteria on a substrate to provide an array of phage infected bacteria;

culturing the phage infected bacteria to produce an array of phage;

making a corresponding copy of the array of phage; and,

treating the array of phage to produce an array of DNA, thereby providing the array of nucleic acids.

13. The method of claim 12, further comprising the step of recovering viable transfected bacteria from the corresponding copy.

14. The method of claim 12, wherein the array of phage is treated with NaOH or EDTA or heat to lyse the phage, thereby releasing nucleic acids of the phage.

15. The method of claim 12, wherein the substrate is selected from the group consisting of nitrocellulose, glass, and plastic.

16. The method of claim 1, wherein the first probe and the second probe are detected optically.

17. The method of claim 16, wherein an optical image of the array is digitized.

18. The method of claim 1, wherein the first probe and the second probe are detected spectrophotometrically.

19. The method of claim 1, wherein the first probe and the second probe are detected with a charged coupled device.

20. The method of claim 1, wherein the first probe and the second probe are detected with a phototube and a photodiode.

21. The method of claim 1, the nucleic acid array comprises between about 100,000 and 1,000,000 species of nucleic acids.

22. The method of claim 1, the nucleic acid array comprises less than 100,000 species of nucleic acids.

23. The method of claim 1, further comprising prehybridizing the array to a non-specific nucleic acid.

24. The method of claim 23, wherein the non-specific nucleic acid is COT 1 DNA.

25. The method of claim 1, further comprising compiling the results of the step of detecting the hybridization of the first and second probes into a database.

26. A method of detecting a chromosomal abnormality comprising:

cloning DNA from a genomic sample into a phage library;

infecting a bacterial culture with the phage library to provide infected bacterial cells;

plating the infected bacterial cells onto an array under conditions permitting replication of phage in the phage library to make an array of phage;

making a corresponding copy of the array of phage to produce a copy array;

treating the phage in the array to produce an array of DNA from the phage to make an array of DNA;

contacting the array of DNA with a first probe having a first color and a second probe having a second color, where the two probes are selected from the group consisting of a fluorescently labeled YAC DNA probe, a fluorescently labeled BAC DNA probe, a spectrally labeled P1 artificial chromosome, a spectrally labeled MAC, a spectrally labeled plasmid, a spectrally labeled phage, and a spectrally labeled cosmid; and,

detecting the two probes.

27. The method of claim 26, wherein proximal hybridization of the two probes to a target DNA from the array produces a third color.

28. An array of nucleic acids comprising:

a plurality of different nucleic acid species on a substrate; and,

at least two probes comprising at least two different fluorescent labels hybridized to at least one nucleic acid species from the plurality of different nucleic acids.

29. The array of claim 28, wherein the two different labels emit different wavelengths of light.

30. The array of claim 29, wherein combination of the different wavelengths provides an optically detectable color signal.

31. An apparatus comprising a solid substrate having at least 10,000 microwells at preselected locations on the solid substrate.

32. The apparatus of claim 31, further comprising nucleic acid in a plurality of said microwells.

33. The apparatus of claim 31, further comprising a plurality of phage in a plurality of said microwells.

34. The apparatus of claim 31, further comprising a plurality of bacteria in a plurality of said microwells.

35. The apparatus of claim 31, wherein the microwells hold between about .1 nanoliters and 10 nanoliters.

36. The apparatus of claim 31, further comprising an armature having a plurality of pins arranged to fit into a plurality of said microwells during operation of the apparatus.

37. The apparatus of claim 31, wherein the substrate comprises at least 100,000 microwells.

38. The apparatus of claim 31, wherein the substrate comprises between about 100,000 and 1,000,000 microwells.

39. The apparatus of claim 31, further comprising detection optics.

40. The apparatus of claim 31, further comprising detection optics which permit simultaneous imaging of the entire substrate.

41. The apparatus of claim 31, further comprising detection optics which permit simultaneous imaging of the entire substrate, which substrate is less than about 100 cm^2 in area.

42. A kit comprising a container, a solid substrate having at least 10,000 microwells at preselected locations on the solid substrate, and instructions for practicing the method of claim 1 using the solid substrate.

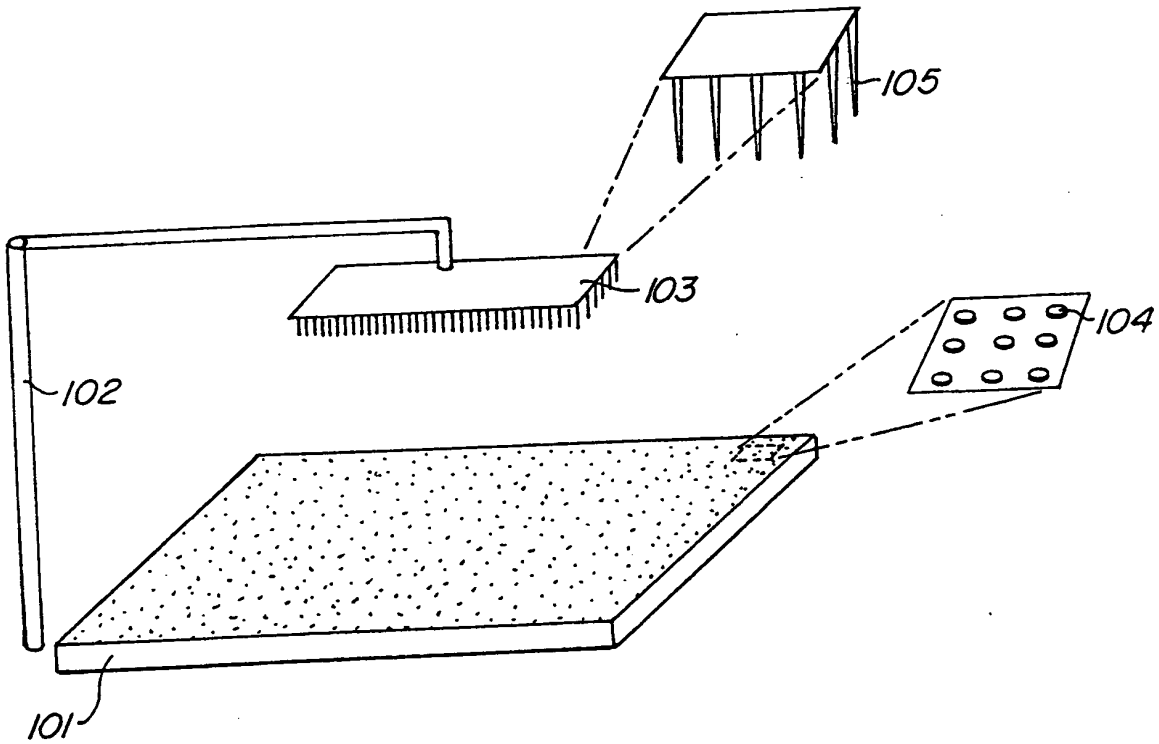

FIG. 1.

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/US 98/13557

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 4 591 567 A (BRITTEN ROY J ET AL) 27 May 1986 see whole document esp. column 6, line 44 ff	31-41 1-30,42
Y	WO 92 05185 A (AMOCO CORP) 2 April 1992 see the whole document	1-30,42
X	WO 96 17958 A (UNIV CALIFORNIA ;MEDICAL RES COUNCIL (GB)) 13 June 1996 see whole document, esp. claims	1-30
X	DE 43 44 726 A (DEUTSCHES KREBSFORSCH) 29 June 1995 see the whole document	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 October 1998	12/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/US 98/13557

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 35505 A (UNIV LELAND STANFORD JUNIOR) 28 December 1995 see the whole document ---	1-42
A	WO 93 10259 A (HOZIER JOHN) 27 May 1993 see the whole document ---	1-42
A	EP 0 709 466 A (GEN PROBE INC) 1 May 1996 see whole document esp. claims -----	1-42

INTERNATIO[illegible] SEARCH REPORT

...formation on patent family members

Inter...al Application No
PCT/US 98/13557

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4591567 A	27-05-1986	NONE	
WO 9205185 A	02-04-1992	AT 148503 T	15-02-1997
		CA 2091764 A	21-03-1992
		DE 69124522 D	13-03-1997
		DE 69124522 T	25-09-1997
		EP 0549709 A	07-07-1993
		EP 0787805 A	06-08-1997
		JP 5501809 T	08-04-1993
		US 5506350 A	09-04-1996
		US 5491224 A	13-02-1996
		US 5789161 A	04-08-1998
		US 5663319 A	02-09-1997
		US 5776688 A	07-07-1998
WO 9617958 A	13-06-1996	EP 0800587 A	15-10-1997
DE 4344726 A	29-06-1995	AU 687493 B	26-02-1998
		AU 1316095 A	17-07-1995
		CA 2179605 A	06-07-1995
		WO 9518235 A	06-07-1995
		EP 0731849 A	18-09-1996
WO 9535505 A	28-12-1995	US 5807522 A	15-09-1998
		AU 2862995 A	15-01-1996
		CA 2192095 A	28-12-1995
		EP 0804731 A	05-11-1997
		JP 10503841 T	07-04-1998
WO 9310259 A	27-05-1993	US 5326691 A	05-07-1994
		AU 3134893 A	15-06-1993
		US 5563060 A	08-10-1996
EP 0709466 A	01-05-1996	AU 4135696 A	23-05-1996
		CA 2201595 A	09-05-1996
		JP 10507351 T	21-07-1998
		WO 9613612 A	09-05-1996
		US 5756709 A	26-05-1998
		US 5658737 A	19-08-1997